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Abstract Cadherins are a family of transmembrane proteins that play a crucial role in cell differentiation, cell migration, and intercellular adhesion. Cadherins are associated with catenins through their highly conserved cytoplasmic domain. Down-regulation of E-cadherin protein has been shown in various human cancers. This study examined the expression of cadherins and associated catenins at the mRNA level. Paired tumor and non-neoplastic primary prostate cultures were obtained from surgical specimens. Quantitative multiplex fluorescence reverse transcriptase-polymerase chain reaction (QMF RT-PCR) and quantitative analysis were performed and correlated with immunostain results. Six of seven cases of neoplastic cultures showed moderately-to-markedly decreased levels of E-cadherin and P-cadherin mRNA. Similar losses of α-catenin and β-catenin mRNA were also observed. The results of QMF RT-PCR showed good correlation with the results of immunohistochemical studies based on corresponding formalin-fixed sections. In conclusion, this paper presents a coordinated down-regulation in the expression of E-cadherin and associated catenins at the mRNA and protein level in most of the cases studied. This down-regulation may play an important role in the pathogenesis of prostate cancer.

Key words Prostate adenocarcinoma · Cadherin · Catenin · Adhesion molecules

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Introduction

Prostate cancer is the most common malignant tumor and the second leading cause of cancer death in men. The clinical course of patients with prostate cancer varies widely, and different factors contribute to this marked clinical variability, including genetic background, hormonal environment, and the invasive potential of the tumor. Invasion and metastasis are the hallmarks of malignancy and have been closely linked to alterations in cell-to-cell adhesion, cell migration, and interactions with extracellular matrix components [22].

Cadherins are a family of transmembrane glycoproteins responsible for maintaining the integrity of tissue and are involved in cell differentiation, cell migration, and intercellular adhesion through a calcium-dependent mechanism characterized by homotypic adhesion [35–37]. Their highly conserved cytoplasmic domains associate with catenins, a group of intracellular proteins that mediate contact between the cadherins and the microfilaments of the cytoskeleton. Each cadherin subclass shows a unique tissue distribution: E-cadherin is predominantly expressed in epithelial cells and P-cadherin is restricted to decidua tissue and the basal or lower layers of stratified epithelium [30].

The accumulating evidence suggests a decrease or loss of function in E-cadherin and P-cadherin in several human carcinomas [3, 9, 23, 32]. Loss of heterozygosity (LOH) at chromosome 16 in the location of the E-cadherin gene is present in a high percentage of prostate cancers [6, 21, 26, 28]. Decreased expression of E-cadherin is seen in various human malignant tumor cell lines, and the level of decrease correlates with the invasive potential of the tumor cell lines [1, 9, 12, 24, 40, 42]. In addition, many, but not all, immunohistochemical studies using formalin-fixed, paraffin-embedded tissue have shown that the E-cadherin protein is decreased in prostate cancer and the decrease is correlated with tumor grade [4, 8, 13, 27, 38]. Other studies have shown decreased-to-absent P-cadherin levels, but variable E-cadherin levels [33].

α- and β-catenins bind the cytoplasmic domain of E-cadherin and link it to the cytoskeleton [16, 31]. Down-regulation of expression and deletion of α-catenin genes were identified in several human cancer cell lines [19, 24, 42]. Immunohistochemical studies showed decreased α-catenin staining, which correlated well with the loss of E-cadherin staining and patient survival [25, 29, 39]. In addition, decreased β-catenin protein expression appears to be associated with malignant transformation of epithelial tissue [34]. These results suggest that cadherins and catenins may function as tumor invasion-suppressor genes. However, most of these results were obtained in studies using transformed tumor cell lines and formalin-fixed, paraffin-embedded tissue. To our knowledge, no studies to date have examined the co-expression of cadherins and catenins at the mRNA level using material derived from surgical specimens.

Recently, we have developed the methodology to cultivate primary epithelial cells under defined conditions from surgical prostatectomy specimens [20]. Areas of both carcinoma and non-neoplastic tissue are identified grossly, verified histologically, and then cultured separately, resulting in paired primary cultures of both non-neoplastic and neoplastic epithelium from the same patient. The non-neoplastic tissue cultures serve as an important control of any person-to-person variability in the expression of the genes of interest.

In this study, we investigated the co-expression of cadherin and catenin mRNA from multiple paired primary prostate cultures derived from surgical prostatectomy specimens using quantitative multiplex fluorescence reverse transcriptase-polymerase chain reaction (QMF RT-PCR) [41, 44]. Immunohistochemistry studies of cadherins and catenins were performed on the corresponding paraffin-embedded prostate tissue.

Materials and methods

Tissue specimen and primary prostate cultures

We studied patients with adenocarcinoma of the prostate who underwent prostatectomy at the University of Pittsburgh Medical Center during the time interval January 1996–January 1998. No patient had received treatment prior to surgery. Fresh prostatectomy specimens were sectioned and grossly examined, representative samples of neoplastic and non-neoplastic tissue were collected, and the diagnoses were confirmed by examination of hematoxylin and eosin (H&E)-stained sections. Epithelial cells from tumor and non-neoplastic areas of the specimen were isolated and then cultured with a chemically defined medium (CDM) without addition of growth factors as previously described. Tissue fragments were cut into small pieces and underwent a series of collagenous digestions. Following each digestion, the cells were pelleted by centrifugation at 1,000 rpm for 4 MIN [20]. To selectively promote epithelial cell growth, the pellets were resuspended and maintained in serum-free CDM supplemented with epithelial growth factor (EGF) [20]. The culture's morphology was examined daily, and epithelial cells were allowed to grow until confluence was reached, between days 7 and 10.

Isolation of total RNA and synthesis of cDNA

Total RNA was extracted from the cultured primary prostate epithelial cells at first passage according to the Trizol solution (Gibco BRL, Rockville, MD) modified method of Chomczynski and Sacchi [7]. The RNA was then quantitated spectrophotometrically. Two micrograms of total RNA were used for first strand cDNA synthesis using oligo-dT primers and MMLV reverse transcriptase (Gibco BRL, Rockville, MD).

PCR primers and quantitative multiplex fluorescence PCR

PCR primers for hepatocyte growth factor (HGF) and c-myc were synthesized as previously described [17]. PCR primers for E-cadherin, P-cadherin, α-catenin, β-catenin, and β-actin cDNA were designed according to cDNA sequences provided by the GeneBank (WWW2.ncbi.nlm.gov/genebank/query). The primers were selected to amplify 150–250 bp target genes and the PCR products from each target gene were designed to have a different size (Table 1). The reverse primers were synthesized with fluorescein molecules covalently attached to the 5' end (BRL, Rockville, MD). Twenty-five microliters of PCR reactions for QMF-PCR contained primers (20 μM each), cDNA corresponding to 50 ng of total RNA, dNTPs, and reaction buffer. The reactions were amplified for 21 cycles at 94 °C for 1 min, 57 °C for 2 min, and 72 °C for 2 min. Five microliters of QMF-PCR reactions were mixed with an equal volume of sequencing gel loading buffer, denatured, and aliquots were electrophoresed on an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA) using a matrix specific for fluorescein (Fig. 1A).

Automated sequencer gels were run for 6 h at 30 W using Genescan software (ABI, Foster City, CA). Lane assignments and areas of the peaks corresponding to fluorescent peaks were assigned and quantitated by the Genescan software using Photomultiplier tube (PMT) voltages (Fig. 1B). All experiments were done in triplicate and the results presented as means and standard deviations (SD).

Antibodies and immunohistochemistry

Five-micron sections were obtained from formalin-fixed, paraffin-embedded tissue blocks. They were deparaffinized and hydrated with graduated ethanols. Slides were then microwaved in 1.1 M

Table 1 Summary of PCR primer sequences and PCR products

Primer	Sequence	Target gene	Size of PCR product (bp)
1	Forward cccacactgtgcccatctacg	β-Actin	170
2	Reverse gcttctccttaatgtcacgc		
3	Forward caaagtgggcatagatggtgtg	E-cadherin	190
4	Reverse ctgcttggatttccagaaacgg		
5	Forward gcaagagccagctctgtttagc	P-cadherin	220
6	Reverse actigagctgattcagtctctgg		
7	Forward gatggacaactatgagccagg	α-Catenin	182
8	Reverse tataccaggcgggaagcatcg		
9	Forward ttctggtgccactaccacagc	β-Catenin	218
10	Reverse tgcatgccctcatctaatgtc		

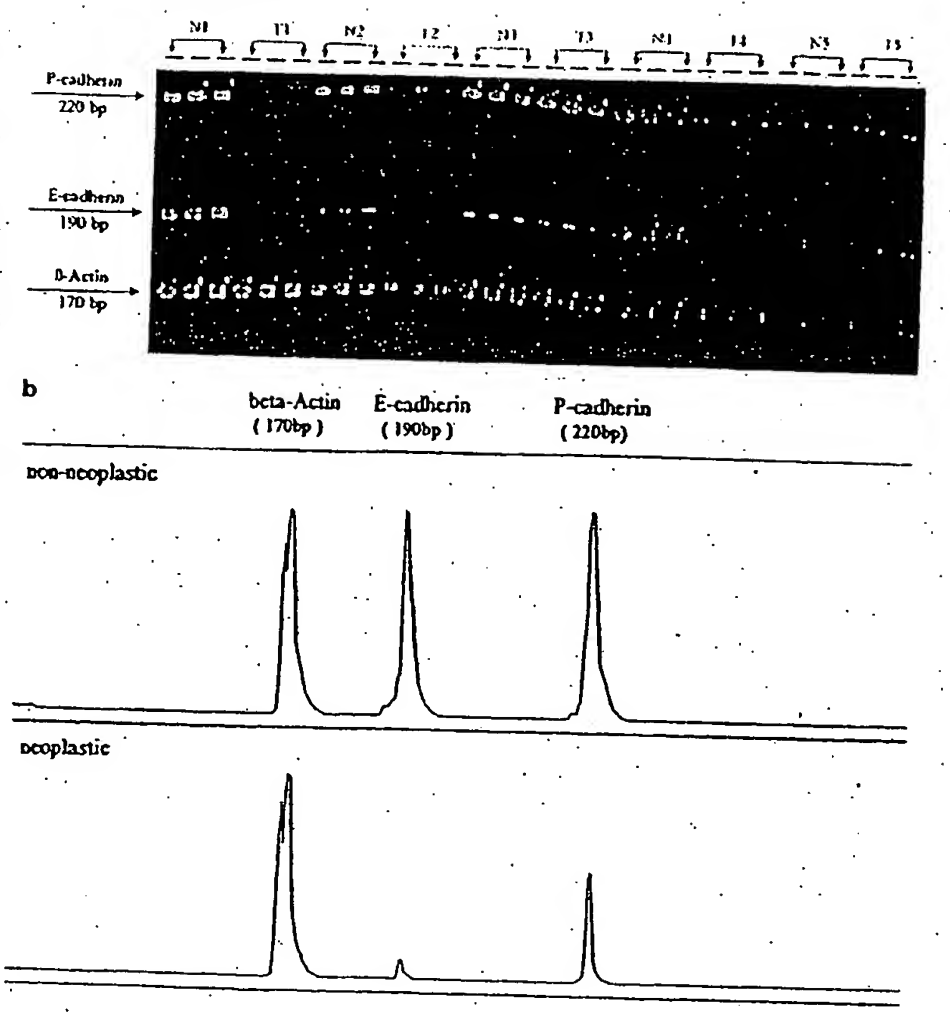

Fig. 1 A Genescan image of QMF RT-PCR of five paired non-neoplastic and neoplastic primary prostate cultures. cDNA corresponding to 50 ng of total RNA was subjected to 21 cycles of PCR, and all reactions were done in triplicate. RNA of both E-cadherin and P-cadherin showed moderate-to-marked reduction in primary neoplastic cultures in four of five cases (Cases 1, 2, 4, and 5). B Representative automated sequencer traces (ABI 373A) from 21 cycles of QMF RT-PCR using cDNA from a paired non-neoplastic (*top panel*) and neoplastic (*bottom panel*) primary prostate cultures (Case 2), and copy number of PCR products (α-actin, E-cadherin, and P-cadherin) was calculated using peak areas. Neoplastic culture showed markedly decreased expression of both E-cadherin and P-cadherin

citrate buffer (pH 6.0) for antigen retrieval. The avidin–biotin complex method for immunohistochemistry was utilized from Vector Laboratories (Burlingame, Calif., USA). The E-cadherin, P-cadherin, α-catenin, and β-catenin antibodies, all mouse monoclonal antibodies, were purchased from Transduction Laboratories (Lexington, Ky., USA).

Evaluation of immunostaining

The percentage of positive cells, intensity of the staining, and cellular localization of the staining were examined by two independent observers using normal prostate epithelium from the same specimen as an internal control. The intensity of the signal was graded as strong, moderate, weak, and negative. The staining pattern of the tumor was compared with that of normal epithelium from the same specimens.

Results

Expression of E-cadherin and P-cadherin

Since many prostate cancers are known to be histologically heterogeneous, adjacent H&E-stained sections of the tissue fragments sent to culture were reviewed to assess tissue homogeneity and to rule out the presence of other diseases. The paired primary cultures for this study were selected based on the following histologic features: (1) non-neoplastic tissue showed no cancerous foci or high-grade prostatic intraepithelial neoplasia (PIN); (2) neoplastic sections contained less than 5% non-cancerous epithelium. Seven out of 38 pairs of cultures met the selection criteria and were included in this study. Histologically, all seven cases were moderately differentiated adenocarcinomas, with Gleason scores ranging from 5 to 7 (median = 6). In six of these cases, adjacent sections of tumor contained no benign prostate epithelium, and in one case (specimen 4), there was less than 5% non-cancerous epithelium. Prostate stromal cells express HGF, whereas the epithelial cells express c-met, the receptor for HGF [17, 20]. The cases included in this study showed no detectable HGF expression after 21 cycles of QMF RT-PCR (data not shown). This indicates there was no significant stromal cell contamination in the current epithelial cultures.

QMF-PCR is an accurate method of measuring the relative levels of mRNA in small tissue samples [41]. In

this study, we used this method to quantitate the mRNA levels of E-cadherin and P-cadherin, relative to β-actin. All the RNA samples contained no DNA contamination, as shown by the absence of automated signals when RT was omitted from the RT-PCR reactions. We observed the expected linear increases in β-actin, E-cadherin, and P-cadherin signal intensities between cycle numbers 18 and 24, with a cDNA input corresponding to 50 ng of total RNA. The ratios of β-actin to E-cadherin and P-cadherin were constant, as was the E-cadherin/P-cadherin ratio. These results indicated that the target genes were consistently amplified in the reactions. Twenty-one cycles of PCR were chosen for all subsequent experiments. In addition, the raw sequencer quantitation of peak areas for β-actin using the same cDNA input was similar among the paired primary prostate cultures (data not shown).

When compared with the paired non-neoplastic primary cultures and normalized with the β-actin internal controls, four of the neoplastic cultures showed marked (>85%) reduction of E-cadherin mRNA levels, with one case showing no detectable E-cadherin mRNA. The other three neoplastic cultures showed mild-to-moderate reductions (Table 2).

Six of seven neoplastic cultures showed moderately to markedly decreased P-cadherin mRNA levels when compared with non-neoplastic cultures. Interestingly, the four cases showing marked reduction in levels of E-cadherin mRNA, and also demonstrated significant losses of P-cadherin mRNA. The case with no detectable E-cadherin mRNA also demonstrated near total loss of P-cadherin mRNA (Case 1). Another case (Case 3), with only mild reduction in E-cadherin mRNA, showed no significant change in P-cadherin mRNA. Genetic variations among the patients were evident in the marked differences in the baseline levels of E-cadherin and P-cadherin expression seen in the non-neoplastic primary prostate cultures.

Expression of α-catenin and β-catenin

The highly conserved intracytoplasmic domain of the cadherins interacts with α- and β-catenins, with the catenins serving as a link between the cadherins and the microfilaments of the cytoskeleton. Six cases of neoplastic cultures showed mild-to-moderate reductions in α-catenin mRNA levels, ranging from 26 to 62%. These cases also demonstrated more severe reductions of β-catenin mRNA levels and generally correlated with the changes of cadherins in the same specimen (Table 3). Interestingly, the case with no significant change of P-cadherin mRNA and only a mild loss of E-cadherin mRNA also showed no change in mRNA levels of both

Table 2 E-cadherin and P-cadherin expression in paired non-neoplastic and neoplastic primary prostate cultures derived from prostatectomy specimens

Case	E-cadherin[a]		Percentage loss in tumor[b]	P-cadherin[a]		Percentage loss in tumor[b]
	Non-neoplastic	Tumor		Non-neoplastic	Tumor	
1	71.0 ± 2.2	ND[c]	100	65.7 ± 2.0	0.8 ± 0.1	99
2	27.4 ± 6.4	3.7 ± 0.3	86	53.4 ± 6.5	17.8 ± 1.3	67
3	18.0 ± 0.2	12.6 ± 1.9	30	88.8 ± 6.5	86.4 ± 9.5	3
4	93.5 ± 12.0	5.4 ± 0.9	94	87.8 ± 7.0	25.5 ± 2.5	71
5	41.5 ± 3.0	24.4 ± 4.0	41	30.5 ± 1.4	18.1 ± 2.4	41
6	56.7 ± 1.1	8.1 ± 1.0	86	71.8 ± 2.9	17.1 ± 2.2	76
7	53.8 ± 6.1	22.2 ± 0.7	59	62.7 ± 3.5	19.4 ± 2.2	69

[a] Level of E-cadherin and P-cadherin are normalized with β-actin from the same sample: (E-cadherin or P-cadherin)/actin × 100
[b] Percentage loss in tumor primary culture: [(N − T)/N] × 100%
[c] ND, not detectable

Table 3 α-Catenin and β-catenin expression in paired non-neoplastic and neoplastic primary prostate cultures derived from prostatectomy specimens

Case	α-catenin[a]		Percentage loss in tumor[b]	β-catenin[a]		Percentage loss in tumor[b]
	Non-neoplastic	Tumor		Non-neoplastic	Tumor	
1	45.1 ± 7.1	17.3 ± 0.5	62	19.7 ± 3.6	ND[c]	100
2	49.2 ± 1.6	36.2 ± 4.0	26	21.7 ± 1.5	3.9 ± 0.2	82
3	48.0 ± 5.9	47.8 ± 9.0	< 1	40.1 ± 5.3	38.9 ± 6.2	3
4	61.1 ± 2.5	40.8 ± 2.6	33	36.8 ± 1.8	22.1 ± 1.9	40
5	58.2 ± 1.8	27.8 ± 1.6	52	29.8 ± 2.0	15.2 ± 1.1	49
6	46.7 ± 4.7	33.6 ± 4.7	28	31.5 ± 4.2	13.5 ± 1.6	57
7	43.2 ± 2.6	26.4 ± 1.7	39	22.3 ± 1.5	11.6 ± 1.4	48

[a] Level of α-catenin and β-catenin are normalized with β-actin from the same sample: (catenin/actin) × 100
[b] Percentage loss in tumor primary culture: [(N − T)/N] × 100%
[c] ND, not detectable

α- and β-catenins. Moderate interspecimen variation was observed in the baseline expression of α-catenin and β-catenin mRNA levels in the non-neoplastic cultures.

Immunohistochemical studies of cadherins and catenins

In benign prostate tissue, E-cadherin was, in all cases, uniformly localized to the membranes of luminal glandular epithelial cells, predominantly at cell–cell junctions (Fig. 3A). One case of prostate cancer showed complete negative staining for E-cadherin (Fig. 3B), and the remaining six cases demonstrated reduced immunostaining for E-cadherin, with 25–75% of cancer cells positive (Fig. 3C). The cancerous glands generally showed reduced signal intensity and an altered heterogeneous staining pattern, which included focal cytoplasmic staining and reduced membranous staining (Fig. 3C, Table 4).

Benign prostate tissue showed uniform basal cell immunoreactivity for P-cadherin, with principally cytoplasmic and focal membranous pattern staining. The benign, glandular non-basal epithelial cells and stromal cells were negative for P-cadherin (Fig. 3D). P-cadherin immunoreactivity was completely absent in two cases of prostate cancer (Fig. 3E), and the remaining five cases showed variable focal positivity, which was predominantly cytoplasmic (Fig. 3F). This focal P-cadherin immunostaining positivity was confirmed by staining multiple sections and by using different monoclonal antibodies. In some cases, serial sections also appeared to show immunostaining for both E- and P-cadherins with the same neoplastic cells.

In all cases of benign prostate tissue, α- and β-catenin protein expression showed strong homogeneous staining of the luminal glandular epithelium and the basal cells. In a pattern similar to that of normal E-cadherin, the α- and β-catenins were localized predominately at luminal epithelial cell–cell borders (Fig. 2A, C). In all cases of prostate cancer, there was a mild-to-moderate reduction in staining for both catenins with 50–75% of cells positive, and the staining tended to be heterogeneous (Fig. 2B, D; Table 4).

Table 4 Immunohistochemical expression of cadherins and catenins in prostatectomy specimens corresponding to primary prostate culture

Case	E-cadherin	P-cadherin	α-catenin	β-catenin
1	−	−	+ +	+ +
2	+ +	+	+ +	+ +
3	+ + +	+ +	+ + +	+ + +
4	+	−	+ +	+ + +
5	+ + +	+	+ + +	+ + +
6	+ +	+	+ + +	+ + +
7	+ +	+	+ +	+ + +

−, Negative; +, <25% positive; + +, 25–50% positive; + + +, 50–75% positive; + + + +, >75% positive

Discussion

In this study, we observed a coordinated down-regulation of the expression in the genes involved in the cadherin and catenin mediated cell–cell pathway at the mRNA level. The protein levels, as demonstrated by the immunohistochemical studies on the corresponding tissue sections, were generally consistent with the mRNA data as well as with that reported in the literature [18, 33, 34, 38, 39].

E-cadherin showed the most consistent loss of expression at both the mRNA level and the protein level.

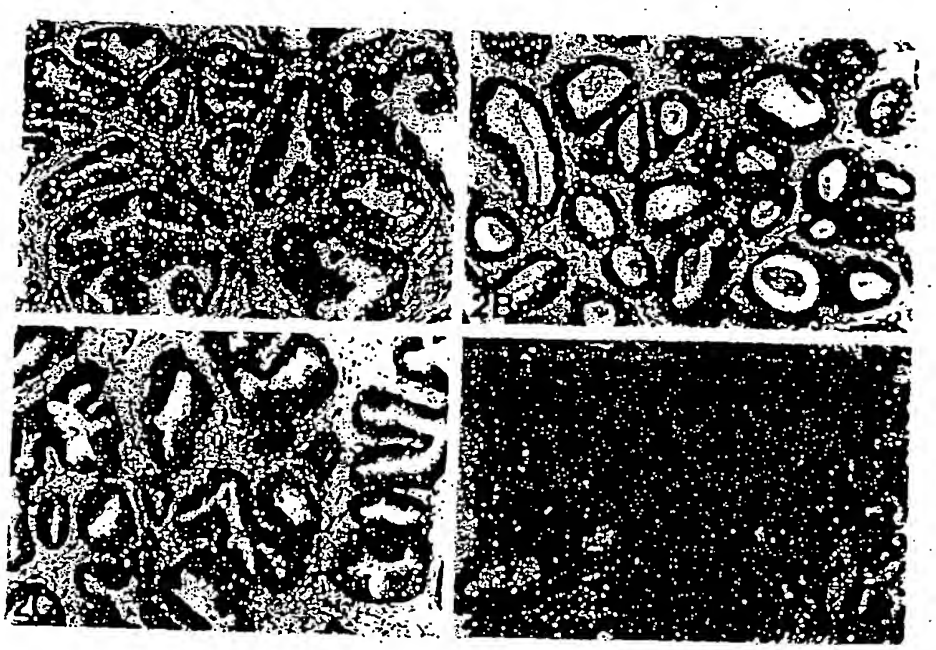

Fig. 2A–D Immunohistochemical staining of α- and β-catenin in non-neoplastic prostate and in prostate adenocarcinoma. Original magnification ×115. A, C Normal membranous expression of α-catenin and β-catenin in non-neoplastic prostatic epithelium (Case 1). B Decreased immunostaining for α-catenin in prostate adenocarcinoma (Case 1). D Decreased and heterogenous immunostaining for β-catenin in prostate adenocarcinoma (Case 1)

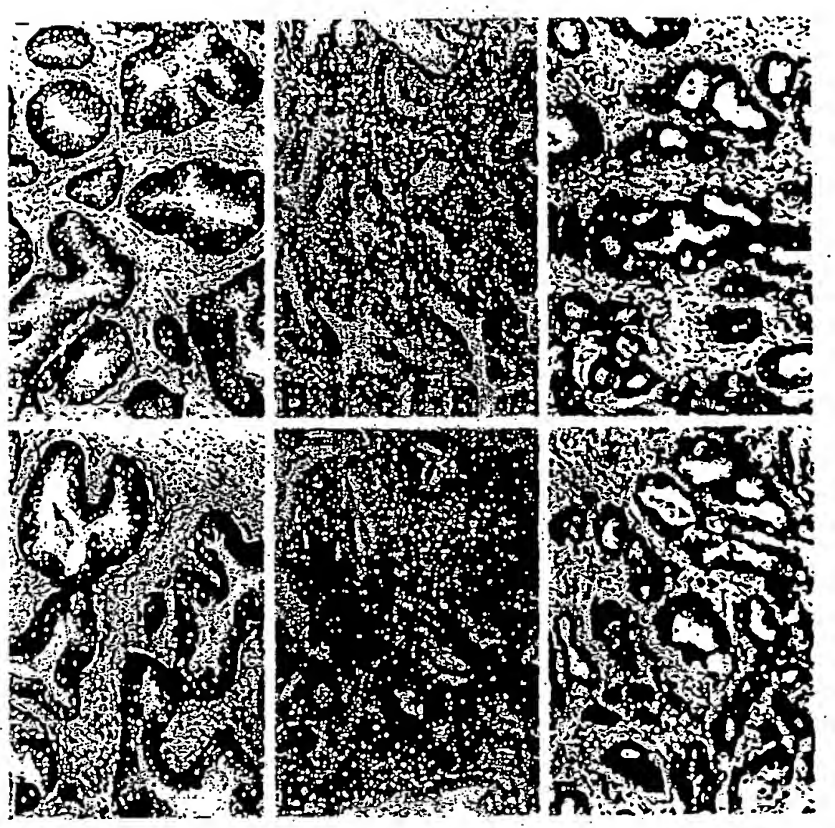

Fig. 3 A Immunohistochemical staining of E- and P-cadherin in non-neoplastic prostate and in prostate adenocarcinoma. Original magnification ×115. A Normal membranous expression of E-cadherin in non-neoplastic prostatic epithelium (Case 1). B, C Complete negative (Case 1) and focal heterogeneous (Case 3) immunostaining for E-cadherin in prostate adenocarcinoma. D Normal continuous basal layer expression of P-cadherin in a portion of non-neoplastic prostatic epithelium (Case 1). E, F Complete negative (Case 1) and mild focal heterogeneous (Case 3) immunostaining for P-cadherin in prostate adenocarcinoma

LOH at chromosome 16q, where both E-cadherin and P-cadherin are located, occurs in up to 30% of prostate cancers. Four of our cases showed a greater than 50% reduction in mRNA, ranging from 87 to 100%, suggesting that mechanisms in addition to LOH may play a role in the reduction of E-cadherin mRNA levels below that of the 50% predicted by LOH alone.

Previous studies have suggested that P-cadherin could serve as a specific marker for basal cell differentiation and was not expressed in prostate cancer, although a recent study has shown focal P-cadherin expression in some prostate tumors [18]. In this study, we demonstrated P-cadherin mRNA in six of seven neoplastic cultures, although it was significantly reduced in all six cases. It is impossible to rule out the possibility that some of the P-cadherin mRNA expression may have resulted from potential contamination by small numbers of non-neoplastic basal cells admixed with the neoplastic cells within the culture material. However, we favor the interpretation that low levels of P-cadherins are expressed in cultured tumor cells, as well as weakly in some tumors in vivo owing to disruption of normal gene regulation. This interpretation is favored by the presence of focal immunostaining for P-cadherin protein in histologically neoplastic cells in five of seven cases. The histologic selection criteria (requiring minimal to no benign prostate glands) should also have minimized major contamination. Despite the presence of mRNA in primary tumor cultures and focal positive immunostaining, P-cadherin immunostaining may still serve as a useful basal cell marker because the staining pattern was distinctly abnormal in the cases where it was focally present.

In this study, the levels of α- and β-catenin mRNA were also lower than normal in six of seven cases, though the reductions were relatively less than those of cadherins, especially for the α-catenins. Catenin protein expression was generally moderate to weak by immunohistochemistry and showed a heterogeneous cytoplasmic and weak membranous staining pattern. The immunopositivity was generally similar in most cases, with 25–75% cells showing positivity. The results of the immunohistochemical studies were generally consistent with the mRNA data; although not in all cases (e.g., Case 1), perhaps due to tumor heterogeneity.

In this study, we also observed a coordinated down-regulation of E-cadherin and the catenins in most cases; this was most observable at the mRNA level. The cadherins are tightly regulated during embryogenesis and appear to serve the need for precise spatio-temporal regulation. The promoter sequences of both E-cadherin and P-cadherin have been cloned and functionally analyzed [2, 5, 10, 15]. Both promoters have similar regulatory elements, such as GC-rich regions and CCAAT boxes. Although the two promoters share similar sequences, in vitro binding studies suggest that the two promoters are regulated by different transcriptional factors [11]. The tissue specificity of these promoters

appears to be derived by different combinations of a relatively few factors common to many types of tissues, and does not appear to be derived from transcription factors specific for each type of tissue. In our study, the degree of loss of expression of α- and β-catenins at the mRNA level was correlated with reductions in the levels of E-cadherin expression. Although the exact mechanism of this coordinated down-regulation is not known, the coordinated pattern supports the hypothesis that loss or alteration of some regulatory factors occurs during prostate tumor progression. Possible mechanisms include transcriptional factor alterations or hypermethylation of the promoter region [14, 43]. Limited information is available on the transcriptional regulation of the catenins, but it is possible that similar mechanisms may play a role.

In summary, this is the first comparative study of the expression of the genes involved in the cadherin-mediated cell–cell adhesion pathway at the mRNA level using paired neoplastic and non-neoplastic primary cultures derived from prostatectomy specimens. Our results indicate that (1) there is a marked patient-to-patient variation in the normal levels of the cadherins and catenins; (2) mRNA levels of E-cadherin as well as catenins are significantly reduced in some prostate cancer primary cultures, and the reduction tends to be to the same degree in each tumor, suggesting a defect in a regulating mechanism common to all of these genes; (3) P-cadherin appears to be present at both the mRNA level and the protein level in some prostate cancers. This coordinated down-regulation of E-cadherin and catenin-mediated adhesion pathways may play a crucial role in tumor pathogenesis and metastasis.

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